Capsule depolymerase overexpression reduces *Bacillus anthracis* virulence

Angelo Scorpio,† Donald J. Chabot, William A. Day,‡ Timothy A. Hoover and Arthur M. Friedlander

United States Army Medical Research Institute of Infectious Diseases, Frederick, MD 21702, USA

Correspondence
Angelo Scorpio
scorpioa@nbacc.net
Arthur Friedlander
arthur.friedlander@us.army.mil

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Capsule depolymerase (CapD) is a γ-glutamyl transpeptidase and a product of the *Bacillus anthracis* capsule biosynthesis operon. In this study, we examined the effect of modulating *capD* expression on *B. anthracis* capsule phenotype, interaction with phagocytic cells and virulence in guinea pigs. Transcriptional fusions of *capD* were made to the genes encoding heat-shock protein 60 (*hsp60*) and elongation factor Tu (*EFTu*), and to *capA*, a *B. anthracis* capsule biosynthesis gene. Translation signals were altered to improve expression of *capD*, including replacing the putative ribosome-binding site with a consensus sequence and the TTG start codon with ATG. CapD was not detected by immunoblotting in lysates from wild-type *B. anthracis* Ames but was detected in strains engineered with a consensus ribosome-binding site for *capD*. Strains overexpressing *capD* at amounts detected by immunoblotting were found to have less surface-associated capsule and released primarily lower-molecular-mass capsule into culture supernatants. Overexpression of *capD* increased susceptibility to neutrophil phagocytic killing and adherence to macrophages and resulted in reduced fitness in a guinea pig model of infection. These data suggest that *B. anthracis* may have evolved weak *capD* expression resulting in optimized capsule-mediated virulence.

INTRODUCTION

Bacillus anthracis is the causative agent of anthrax and is considered one of the most important biological warfare threats. The virulence of *B. anthracis* is primarily attributed to genes located on pathogenicity islands of pX01 and pX02. A tripartite toxin composed of protective antigen, lethal factor and oedema factor is encoded on pX01, while genes for the synthesis of an antiphagocytic, poly-γ-D-glutamic acid capsule are carried on pX02 (Mock & Fouet, 2001). The capsule operon is composed of five genes, *capB,C,A,D* and *E*. Capsule depolymerase, encoded by *capD*, is a γ-glutamyl transpeptidase (GGT)-family protein that autocatalytically forms a heterodimer consisting of 35 kDa and 15 kDa subunits. CapD shares 32 % identity with the *Bacillus subtilis* GGT and 35 % identity with a recently reported *Francisella* CapD homologue (Su *et al.*, 2007). Despite this amino acid sequence similarity, the *B. subtilis* GGT is reported to be secreted (Kimura *et al.*, 2004), while *B. anthracis* CapD is membrane associated (Candela & Fouet, 2005). The individual subunits of CapD associate to form a functional enzyme that cleaves the γ-linked peptide bond of the polyglutamate capsule, transferring glutamate to a water molecule (hydrolysis) or an amino acid (transpeptidation). This mechanism results in release of low-molecular-mass capsule and attachment of the capsule to the peptidoglycan layer (Candela & Fouet, 2005; Makino *et al.*, 2002; Uchida *et al.*, 1993), specifically, to the side-chain amino group of *meso*-diaminopimelic acid (Richter *et al.*, 2009). We previously demonstrated that the capsule-degrading activity of recombinant CapD can be exploited to enzymically remove the capsule from the surface of bacilli, resulting in enhanced phagocytic killing by human neutrophils and protection in mouse models of anthrax infection (Scorpio *et al.*, 2007, 2008).

We observed by proteomic analysis that CapD, like CapB, C and A, is associated with the membrane during growth in laboratory media and *in vivo* (Scorpio *et al.*, 2005). An interesting finding from this study was that while CapB, C and A were detected in similar amounts, CapD was only weakly detected in whole-cell and membrane-enriched preparations from laboratory-grown bacilli and was detected in amounts <1 % that of CapB, C and A in bacilli isolated from infected guinea pigs. Nevertheless, a functional CapD has been shown to be required for virulence in mice and guinea pigs (Candela & Fouet, 2005; Makino *et al.*, 2002; Richter *et al.*, 2009). These observations led us to hypothesize that increased expression of *capD* may result in more capsule being released from the

†Present address: Battelle National Biodefense Institute, Frederick, MD 21702, USA.

‡Present address: Ventana Medical Systems, Tucson, AZ 85755, USA.

Abbreviation: GGT γ-glutamyl transpeptidase.

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14. ABSTRACT

Capsule depolymerase (CapD) is a c-glutamyl transpeptidase and a product of the Bacillus anthracis capsule biosynthesis operon. In this study, we examined the effect of modulating capD expression on B. anthracis capsule phenotype, interaction with phagocytic cells and virulence in guinea pigs. Transcriptional fusions of capD were made to the genes encoding heat-shock protein 60 (hsp60) and elongation factor Tu (EFTu), and to capA, a B. anthracis capsule biosynthesis gene. Translation signals were altered to improve expression of capD, including replacing the putative ribosome-binding site with a consensus sequence and the TTG start codon with ATG. CapD was not detected by immunoblotting in lysates from wild-type B. anthracis Ames but was detected in strains engineered with a consensus ribosome-binding site for capD. Strains overexpressing capD at amounts detected by immunoblotting were found to have less surfaceassociated capsule and released primarily lower-molecular-mass capsule into culture supernatants. Overexpression of capD increased susceptibility to neutrophil phagocytic killing and adherence to macrophages and resulted in reduced fitness in a guinea pig model of infection. These data suggest that B. anthracis may have evolved weak capD expression resulting in optimized capsule-mediated virulence.

15. SUBJECT TERMS

Bacillus anthracis, anthrax, capsule depolymerase, overexpression, virulence

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surface of bacilli, potentially allowing the bacilli to be more readily phagocytosed. To investigate this, in the present study we upregulated *capD* expression using transcriptional fusions to various promoters and modifications to translation signals. Strains with higher *capD* expression had less surface-associated capsule, increased susceptibility to phagocytic killing, and reduced virulence in guinea pigs compared with strains with wild-type levels of *capD* expression. We propose that *B. anthracis* may have evolved weak *capD* expression signals resulting in wild-type amounts of cell-associated capsule and a ratio of low- to high-molecular-mass capsule that is optimal for virulence.

METHODS

Bacterial strains and animals. Strains and plasmids used in this study are listed in Table 1. *B. anthracis* Ames (pX01$^+$ pX02$^+$) and ΔANR (pX01$^-$ pX02$^-$) were cultured in brain heart infusion (BHI) broth with 0.8 % sodium bicarbonate and 5 % carbon dioxide or on NBY bicarbonate agar plates at 37 °C with 5 % carbon dioxide (Chabot *et al.*, 2004). *Escherichia coli* strains DH5α and GM2163 were used for cloning and strain construction. Antibiotics were used at the following concentrations: kanamycin, 50 μg ml^{-1} for *E. coli* strains and 20 μg ml^{-1} for *B. anthracis*; and spectinomycin, 200 μg ml^{-1} for *B. anthracis*. *B. anthracis* spores were prepared as previously described (Chabot *et al.*, 2004). Female Hartley guinea pigs, 350–400 g, were obtained from Charles River Laboratories. Female Swiss Webster mice, 6–8 weeks old, were obtained from the National Cancer Institute.

Construction of a *capD* knockout strain of *B. anthracis* Ames. All but the first 180 and last 105 bases of *capD* were deleted by homologous recombination with plasmid pE03 (Mendelson *et al.*, 2004) containing the promoterless *aphA*-3 cassette from pUC18k (Menard *et al.*, 1993) flanked by regions upstream and downstream of *capD*. The upstream flank was made by PCR with a 5′ *Not*I site using forward primer 5′-ATAAGAATGCGGCCGCGAAGCTGATCTTGA-CTATGTGGGTGCTGGTG-3′ and reverse primer 5′-TCCCCCG-GGTCAACCGCAAGGGGGTGAGAGGCACTCACTC-3′. The downstream flank was made by PCR with a 3′ *Kpn*I site with forward primer 5′-TCCCCCCGGGGCAGAAGAAATGGAACTTGGAAATC-AAATAAATAGGAGG-3′ and reverse primer 5′-GGGGTACCCC-AGTAATTAAGACACCGTCAAATCCGT TCTAC-3′.

Flanks were cut with the appropriate enzyme plus *Xma*I and ligated simultaneously into pE03 after digestion with *Kpn*I and *Not*I. The *aphA*-3 nonpolar mutagenesis cassette from pUC18k was then cloned into the *Xma*I site. Selection for homologous recombination was performed as described previously (Mendelson *et al.*, 2004). Stable knockouts were selected for by loss of erythromycin resistance (5 μg ml^{-1} in LB broth) and were confirmed by PCR with internal and external primers. Colonies appeared hypermucoid, compared to wild-type, when grown on NBY bicarbonate plates at 37 °C with 5 % CO_2.

Construction of *capD* fusion strains. Transcriptional fusions of *capD* were created by cloning an approximately 300 bp PCR product encoding the 3′ end of *hsp60* (BA0267), *EFTu* (BA0108) or *capA* into the shuttle plasmid pASD2 (Day *et al.*, 2007) as an *Xma*I–*Xho*I fragment. The ORF of *capD* including 29 bp upstream from the putative start codon was cloned 3′ to the above fragments as an *Xho*I–*Kpn*I fragment. The reverse primer used to generate *capD* for fusion to *capA* was designed to anneal at the 3′ end of *capE*. Additional PCR products were generated that included the *capD* ORF and contained a consensus ribosome-binding site (RBS), AGGAGGT, and a TTG start codon with the forward primer 5′-GCGGCGCTCGAGAGGAGGT-CAACAACTTGAATTCCTTTAAATGGGGAAAGA-3′ and the above *capD* reverse primers. Additional *capD* ORFs were generated that contained a consensus RBS and an ATG start codon or the wild-type RBS and a TTG start codon. Primers employed to generate the amplicons are listed in Table 2. The pASD2 plasmid constructs were passaged through *E. coli* GM2163 before electroporation into *B. anthracis* strains. *B. anthracis* ΔANR (pX01$^-$ pX02$^-$) was transformed with the plasmids containing *capD* fused to *hsp60* or *EFTu*, and kanamycin/spectinomycin-resistant colonies were isolated on Luria–Bertani (LB) agar containing antibiotics. The plasmids were integrated at the 3′ end of *hsp60* or *EFTu* as previously described (Day *et al.*, 2007) and the linkages transferred to fully virulent *B. anthracis* Ames by CP-51 phage transduction (Green *et al.*, 1985). This resulted

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
B. anthracis		
Ames	Wild-type	Lab collection
ΔANR	pXO1$^-$ pXO2$^-$	Lab collection
E. coli		
DH5α		New England Biolabs
GM2163	*dam dcm*	New England Biolabs
Plasmids		
pE03	Integrative plasmid	Mendelson *et al.* (2004)
pASD2	Integrative plasmid	Day *et al.* (2007)
pASD2*hsp60capD*	Fusion of the 3′ end of *hsp60* to *capD* ORF	This work
pASD2*hsp60capD*RBS	Fusion of the 3′ end of *hsp60* to *capD* ORF with engineered RBS for *capD*	This work
pASD2*EFTucapD*RBS	Fusion of the 3′ end of *EFTu* to *capD* ORF with engineered RBS for *capD*	This work
pASD2*capAcapD*RBSATG	Fusion of the 3′ end of *capA* to *capD* ORF with engineered RBS and ATG *capD* start codon	This work
pASD2*capAcapD*TTG	Fusion of the 3′ end of *capA* to *capD* ORF with engineered TTG *capD* start codon	This work
pASD2*capAcapD*RBSTTG	Fusion of the 3′ end of *capA* to *capD* ORF with engineered RBS and TTG start codon	This work
pASD2*capAcapD*ATG	Fusion of the 3′ end of *capA* to *capD* ORF with engineered ATG *capD* start codon	This work
pASD2*capAcapD*	Fusion of the 3′ end of *capA* to *capD* ORF	This work

Table 2. Oligonucleotides used to create amplicons for construction of *capD* fusion strains

F, Forward primer; R, reverse primer.

PCR product	Primer sequences
hsp60 3′ end	F: 5′-GCGGCGCCCGGGTAGAAGAGCCAGTTC GTCAAATCGCAATCA
	R: 5′-GCGGCGCTCGAGTTACAT CATTCCGCCCATACCGCCCATGCC
EFTu 3′ end	F: 5′-GCGGCGCCCGGGCGTATTATCTAAAGAAGAAGGTGGACG
	R: 5′-GCGGCGCTCGAGTTACTCAACGATAGTAGCAACTACACC
capA 3′ end	F: 5′-GCGGCGCCCGGGAGTGCACTTGTGCAATATCATTTACGTGAT
	R: 5′-GCGGCGCTCGAGTCAAGTTGTTGTCTCCACTGATACTTG ATT
capD ORF	F: 5′-GCGGCGCTCGAGGAAAATCAAGTATCAGTG GAGACAACAACTTGA
	R: 5′-GCGGCGGGTACCCTATTT ATTTGATTTCCAAGTTCCATT
	R: 5′-GCGGCGGGTACCTTAGGGGTTAGCCTGTAGATAATCACTAAT*

*Reverse primer used to generate *capD* ORF for fusion to *capA*.

in two copies of *capD* in the *hsp60* and *EFTu* fusions, one on the chromosome and one on pX02. The fusions of *capD* to *capA* were made by integrating the fusion plasmids at the 3′ end of *capA* in the *B. anthracis* Ames *capD* mutant. Genetic linkages were confirmed by dye terminator Sanger sequencing.

Analysis of CapD and capsule expression. Strains overexpressing *capD* and wild-type Ames were grown in BHI/bicarbonate broth at 37 °C with shaking. Bacteria were collected by centrifugation and resuspended in PBS, pH 7.4. The cells were disrupted by sonication and the resulting lysates added to an equal volume of 2 × SDS protein sample buffer. Samples were heated at 100 °C for 10 min and centrifuged for 2 min. The extracted proteins were separated by SDS-PAGE and stained with Gel Code Blue (Pierce Biotechnology). Expression of *capD* was examined by immunoblotting (Invitrogen) and probed with mouse polyclonal anti-CapD antiserum at 1 : 1000 dilution. Capsule in culture supernatants from *capD*-overexpressing strains was visualized by electrophoresis in 1 % agarose gels followed by staining with 0.3 % methylene blue in 20 % ethanol and destaining with water. In one experiment 9 µl of the culture supernatant from *EFTucapD*RBS containing low-molecular-mass capsule was treated with 1 µl (0.3 µg) recombinant CapD (prepared as described previously: Scorpio *et al.*, 2007) and 1.1 µl 1 M serine. Mixtures with and without CapD were incubated at 37 °C for 2 h. Capsule degradation by CapD was visualized by electrophoresis in 2 % agarose gels followed by staining with methylene blue, as described above.

Neutrophil killing and macrophage adhesion assays. Neutrophils were isolated and killing assays performed as previously described (Scorpio *et al.*, 2007). Briefly, heat-shocked spores were germinated (2×10^{7} ml^{-1}) in BHI containing 0.8 % sodium bicarbonate for 90–120 min until capsule was readily visible by India ink stain. Encapsulated bacilli were resuspended in Dulbecco's Modified Eagle's medium containing 10 % heat-inactivated fetal calf serum (DF10) and 10 % type AB human serum (Sigma Aldrich) as a source of complement. Purified human neutrophils were mixed with bacilli in duplicate at an effector : target ratio of 50 : 1 and rotated for 5 h. Bacterial viability was measured by serial dilution in water and plating on LB agar. Results are given as the mean ± SEM of the duplicate samples. Murine RAW264.7 macrophages were grown and used in an adhesion assay as previously described (Scorpio *et al.*, 2007). Briefly, macrophages were allowed to adhere to circular coverslips overnight or until growth to confluence. Spores were germinated in BHI/bicarbonate, the bacilli resuspended in DF10 at a concentration of 1×10^{8} ml^{-1} and layered onto the macrophages in a volume of 200 µl in duplicate. After 30 min of incubation at 37 °C, the coverslips were washed extensively with PBS and stained with Wright–Giemsa. Bacilli adherent to macrophages were counted visually from at least 10 fields of view (at least 100 macrophages) on duplicate coverslips and the mean number of bacilli per macrophage (± SEM) determined as previously described (Scorpio *et al.*, 2007).

CapD antibody production. Mice were vaccinated subcutaneously with 10 µg recombinant CapD (Scorpio *et al.*, 2007) in 200 µl PBS and Ribi R700 adjuvant (Sigma Aldrich), as recommended by the manufacturer. Mice were boosted at 4 weeks and bled from the periorbital sinus at 7 weeks. Blood was collected in serum separator tubes and sera were withdrawn after microcentrifugation for 20 min at 11 000 r.p.m.

Virulence determination. Spores for all challenge experiments were heat-shocked for 40 min at 65 °C, resuspended in water for injection and administered by intramuscular injection in 200 µl. Competitive indices were determined by infecting guinea pigs with a mixture containing equal numbers of spores derived from wild-type *B. anthracis* Ames and a *capD*-overexpressing strain. Guinea pigs were monitored for signs of infection, and when moribund (40–48 h after infection) were euthanized and the spleens removed. The spleens were disrupted by mashing with a 10 ml syringe plunger in a 70 µm filter basket placed in a 50 ml centrifuge tube. Spleen cells were washed through the basket with cold PBS. Relative numbers of the wild-type Ames and the *capD*-overexpressing strain were determined by plating serial dilutions on LB agar containing or lacking antibiotics. Virulence was also determined in guinea pigs by challenge with an intramuscular injection of 2000 spores derived from either wild-type Ames or a *capD*-overexpressing strain and comparing mean time-to-death.

Statistics. Neutrophil killing and macrophage adherence comparisons were evaluated by analysis of variance (ANOVA) with Tukey's post hoc tests. Differences in virulence were evaluated with Student's *t*-test for competitive index and log rank test for survival curve.

RESULTS

Translational signalling affects *capD* expression

To examine the effect of transcriptional and translational signalling on *capD* expression, the *capD* ORF and

translation initiation site were cloned into pASD2 downstream of the 3′ end of *hsp60*, *EFTu* or *capA* and the resulting plasmids integrated via homologous recombination at the respective genes in *B. anthracis* Ames. Two transcriptional fusions of *capD* were made to *hsp60*, one carrying the wild-type *capD* RBS and one with a consensus RBS (AGGAGGT). One transcriptional fusion was made to *EFTu* with a consensus RBS for *capD* translation. Five transcriptional fusions of *capD* were made to *capA* consisting of combinations of an ATG or TTG start codon with the wild-type or consensus RBS. According to the most recent *B. anthracis* genome annotation (Ames ancestor), the stop codon of *capA* overlaps with the TTG start codon of *capD* (http://www.ncbi.nlm.gov/nuccore/NC_007323). Construction of the *capAcapD* fusions carrying a consensus RBS resulted in a 20 bp separation between the *capA* stop codon and the *capD* start codon. The *capAcapD* fusion strains constructed to have TTG or ATG start codons and wild-type RBS had two possible translation start sites separated by 36 bp. The *capAcapD* fusions included *capE*, which has been shown to be necessary for capsule synthesis (Candela *et al.*, 2005), whereas *capE* was not included in *capD* fusions to *hsp60* and *EFTu*, which were made in Ames carrying a functional *capE* on pXO2. Genetic linkages of the *capAcapD* fusion strains are detailed in Table 3.

Expression of *capD* was examined by immunoblotting of whole-cell lysates from strains grown for 5 h or overnight (18 h) at 37 °C in BHI/bicarbonate broth. Consistent with our observations that CapD is poorly detected by proteomics analysis, we were unable to detect CapD in lysates of either the parent wild-type Ames strain or a *capD* mutant at 18 h (Fig. 1a) or 5 h (Fig. 1c). By contrast, CapD was readily detected in lysates of *hsp60capD*RBS and *EFTucapD*RBS, in which the predicted *capD* RBS was replaced with a consensus RBS sequence. CapD was also detected at 5 h and 18 h in *capAcapD*RBSATG, which contains both a consensus RBS and an ATG start codon (Fig. 1a, c). CapD was not detected in *capAcapD*, in which the pASD2 *capAcapD*RBSATG plasmid integrated at *capD* directly after the engineered ATG start codon and formed a wild-type linkage of *capD* to *capA*. This strain behaved identically to wild-type Ames in all experiments. Additionally, CapD was not detected in *capAcapD*RBSTTG, *capAcapD*TTG or *capAcapD*ATG (Fig. 1a), suggesting that both a consensus RBS and an ATG start codon were necessary for detectable *capD* expression for *capAcapD* fusions. Interestingly, for all lysate preparations tested, only the large subunit of CapD reacted with the polyclonal antibody, suggesting that the small subunit was poorly immunogenic, consistent with a previous report (Candela & Fouet, 2005).

capD expression affects *B. anthracis* phenotype

The effect of *capD* overexpression on the phenotype of *B. anthracis* was examined by germinating spores in BHI broth containing 0.8 % bicarbonate under 5 % CO_2 and growing them for 5 h or overnight at 37 °C. Bacilli were visualized by India ink staining and phase-contrast microscopy. When grown in BHI broth, fusions of *capD* to *hsp60* and *EFTu* in which a consensus RBS was present appeared significantly less encapsulated than the parent wild-type Ames strain, while the *capAcapD*RBSATG fusion appeared devoid of capsule (Fig. 2). By contrast, *capAcapD*, *capAcapD*TTG and *capAcapD*ATG bacilli were encapsulated and appeared similar to wild-type Ames by India ink staining, while *capAcapD*RBSTTG was slightly less encapsulated (data not shown).

Agarose gel electrophoresis of BHI/bicarbonate culture supernatants revealed that high-molecular-mass capsule was readily visible in the supernatant from the parent wild-type Ames strain and from *capAcapD* but was not visible in any of the *capD*-overexpressing strains, *capAcapD*RBSATG, *EFTucapD*RBS and *hsp60capD*RBS (Fig. 1b). Intermediate-sized capsule was observed in culture supernatants from *hsp60capD*, while almost entirely low-molecular-mass capsule was observed from *capAcapD*RBSATG, *EFTucapD*RBS and *hsp60capD*RBS. Strain *capAcapD*TTG appeared to produce capsule of a slightly higher molecular mass than wild-type Ames,

Table 3. Nucleotide linkages of *capAcapD* fusion strains

RBSs are underlined and the *capD* start codon and *capA* stop codon are underlined and in bold.

Strain	*capAcapD* linkage
Wild-type	TATCAGT<u>GGAG</u>ACAACAAC<u>**TTGA**</u>ATTCCTTTAAA
*capAcapD*RBSATG	TATCAGT<u>GGAG</u>ACAACAAC<u>**TTGA**</u>CTCGAG<u>AGGAGGT</u>CAA CAAC<u>**ATG**</u>AATTCCTTTAAA
*capAcapD*RBSTTG	TATCAGT<u>GGAG</u>ACAACAAC<u>**TTGA**</u>CTCGAG<u>AGGAGGT</u>CAA CAAC<u>**TTG**</u>AATTCCTTTAAA
*capAcapD*ATG	TATCAGT<u>GGAG</u>ACAACAAC<u>**TTGA**</u>CTCGAGGAAAATCAAG TATCAGT<u>GGAG</u>ACAACAAC<u>**ATG**</u>AATTCCTTTAAA
*capAcapD*TTG	TATCAGT<u>GGAG</u>ACAACAAC<u>**TTGA**</u>CTCGAGGAAAATCAAG TATCAGT<u>GGAG</u>ACAACAAC<u>**TTG**</u>AATTCCTTTAAA

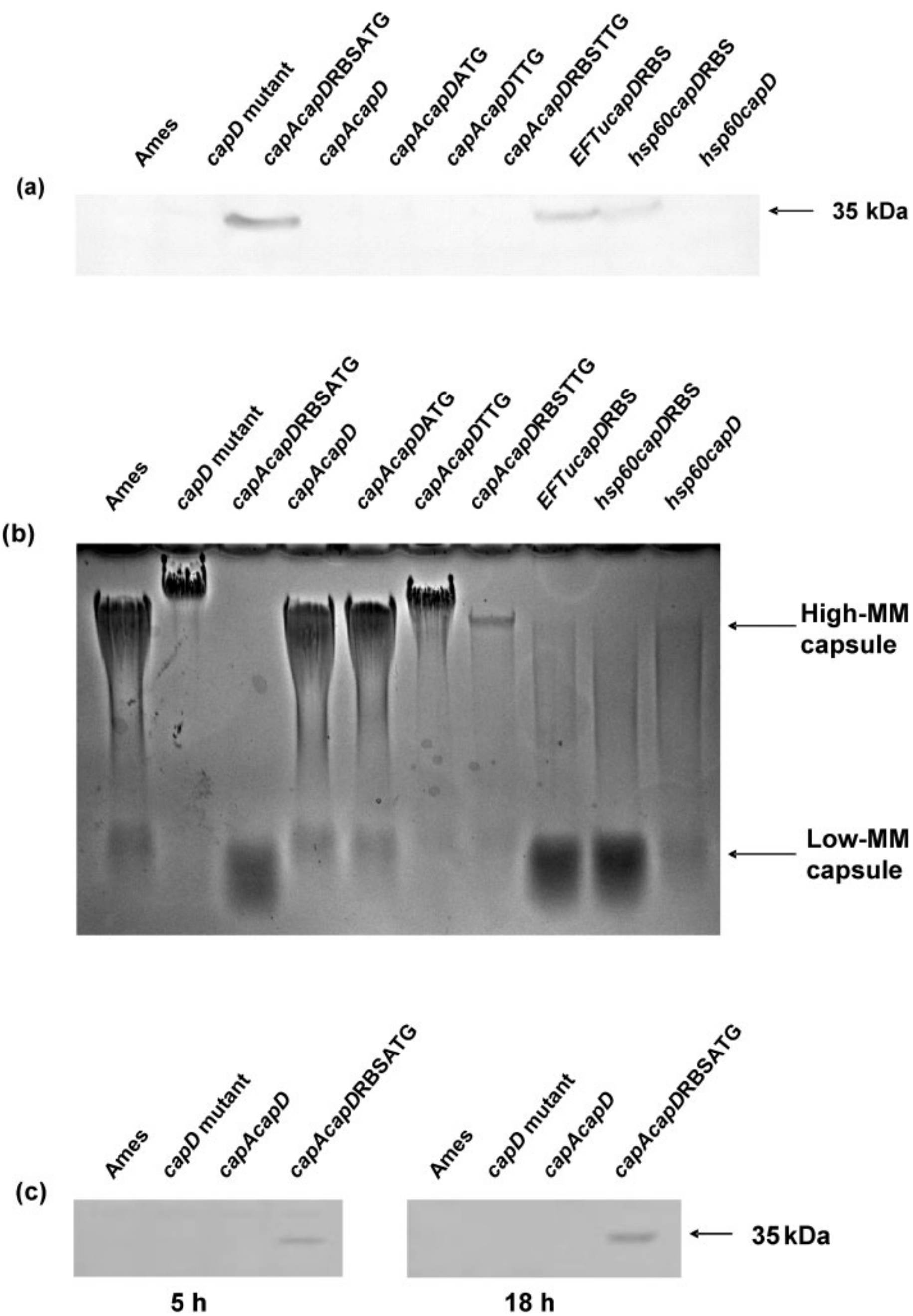

Fig. 1. Effect of *capD* linkage on *capD* expression and capsule phenotype. (a) Whole-cell lysates were made for each strain. An equal amount of protein for each sample was separated by electrophoresis in a 4–12 % SDS-Tricine gel and CapD was detected by immunoblotting using a polyclonal mouse anti-CapD serum. (b) Strains were grown in BHI/bicarbonate broth and capsule in the supernatant was examined by agarose gel electrophoresis and staining with methylene blue. Lane identities are identical to (a). (c) Temporal expression of *capD* determined by immunoblotting of whole-cell lysates 5 or 18 h after germination.

suggesting a lower level of *capD* expression, while unexpectedly, *capAcapD*RBSTTG appeared to produce less total capsule than wild-type Ames (Fig. 1b). The poly-D-glutamic acid nature of the low-molecular-mass capsule was confirmed by reacting it with recombinant CapD and observing degradation on agarose gels (Fig. 3) as described previously for high-molecular-mass capsule (Scorpio *et al.*, 2007).

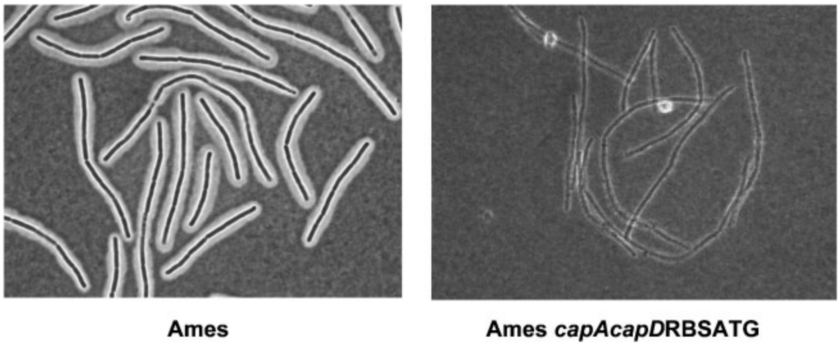

Fig. 2. India ink stain of bacilli from *B. anthracis* Ames and *capAcapD*RBSATG grown overnight in BHI/bicarbonate and examined by phase microscopy (magnification ×700).

Overexpression of *capD* reduces *B. anthracis* virulence

The effect of overexpressing *capD* on *B. anthracis* virulence was examined in cell culture and in a guinea pig infection model. Bacilli from two strains which overexpressed *capD*, *capAcapD*RBSATG and *EFTucapD*RBS, strongly adhered to RAW264.7 murine macrophages, while wild-type Ames failed to adhere (Table 4, $P<0.001$, ANOVA with Tukey's post hoc tests). In addition, bacilli from strains *capAcapD*RBSATG and *EFTucapD*RBS were susceptible to neutrophil killing, with >99 % reduction in viability compared to wild-type Ames bacilli, which were resistant, showing slight growth during the incubation (Table 4, $P<0.001$, ANOVA with Tukey's post hoc tests) as previously reported (Scorpio *et al.*, 2007). Wet-mount microscopy of bacilli and neutrophil mixtures revealed bacilli from *capD*-overexpressing strains engulfed in phagocytic vacuoles while bacilli from wild-type Ames were almost entirely extracellular (data not shown).

Virulence in guinea pigs was measured by intramuscular injection of wild-type Ames and *capAcapD*RBSATG spores in a 1 : 1 ratio (1000 spores of each strain). Animals were euthanized when moribund and the amount of each strain was measured by plating serial dilutions of spleen homogenate onto LB agar with and without antibiotics. Differences in the mean c.f.u. per g spleen between the two strains revealed that *capAcapD*RBSATG was significantly under-represented relative to wild-type Ames (Fig. 4a, $P=0.011$, Student's *t*-test, $n=4$). To examine virulence in a non-mixed infection, guinea pigs were infected with 2000 spores of either *capAcapD* or *capAcapD*RBSATG and monitored for mortality. Strain *capAcapD* was chosen for comparison as it contains the pASD2 plasmid integrated at *capD* but is phenotypically identical to wild-type Ames, suggesting that the integrated plasmid does not have polar effects downstream that may inhibit capsule synthesis. All animals succumbed or were euthanized after infection with both strains. Infection with *capAcapD*RBSATG resulted in a mean time to death of 54.4 ± 3.6 h ($n=5$) compared with 40.5 ± 1.0 h ($n=4$) for *capAcapD*, representing a significant increase in survival (Fig. 4b, $P=0.0027$, log rank test). By comparison, previous experiments performed with a 2000

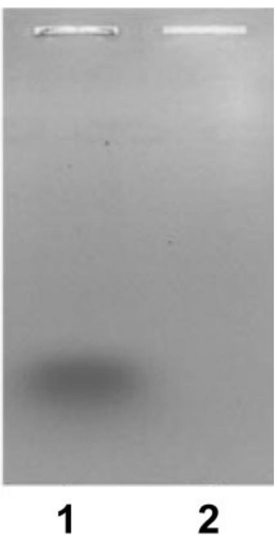

Fig. 3. Low-molecular-mass capsule can be degraded by CapD. *EFTucapD*RBS supernatant was digested for 2 h at 37 °C with 0.3 μg CapD in PBS with 0.1 M serine (lane 2). Undigested capsule was treated in the same way, except that CapD was not included (lane 1). Samples were electrophoresed in 2 % agarose and the gel stained with methylene blue.

Table 4. Macrophage adherence and neutrophil killing of *capD*-overexpressing strains

Bacilli from the different strains were assayed for macrophage adherence and neutrophil killing as described in Methods.

Strain	Macrophage adherence (no. of bacilli per cell, mean ± SEM)	Neutrophil killing (% viable, mean ± SEM)
Ames	0.04 ± 0.03	181.3 ± 86
*EFTucapD*RBS	3.9 ± 1.4*	0.04 ± 0.01*
*capAcapD*RBSATG	1.9 ± 0.4*	0.01 ± 0.001*

*$P<0.001$ vs wild-type Ames.

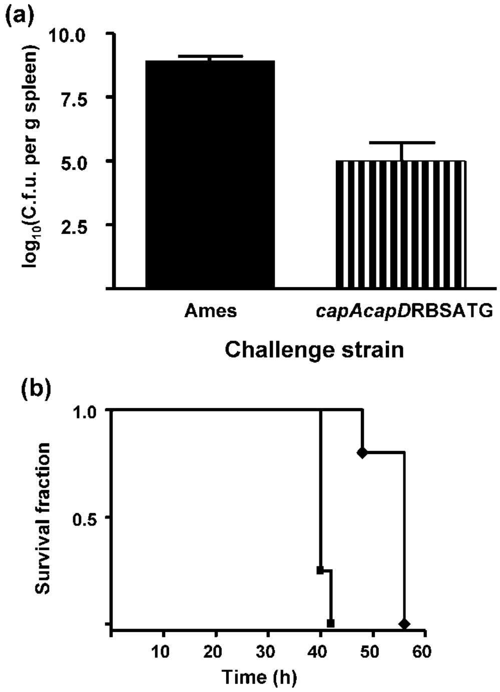

Fig. 4. Effect of *capD* expression on virulence in guinea pigs. (a) Competitive index of *capAcapD*RBSATG vs Ames. Guinea pigs were challenged with a mixture containing equal numbers of heat-shocked spores derived from Ames or *capAcapD*RBSATG. The number of c.f.u. g^{-1} from the spleens was counted for each strain by plating on antibiotic-selective medium (P=0.011). Data are presented as log c.f.u. per g spleen. (b) Non-mixed infection. Guinea pigs were infected with 2000 spores of either *capAcapD* (■) or *capAcapD*RBSATG (◆) and monitored for mortality. Analysis of Kaplan–Meier survival curves showed that the probability of survival was significantly different (P=0.0027).

spore Ames challenge resulted in a mean time to death of 43.7±3.3 h (unpublished observations).

DISCUSSION

B. anthracis grows to high numbers in the blood and organs of infected animals. This high-density growth is largely a result of resistance to innate immune defence mechanisms conferred in part by the antiphagocytic polyglutamate capsule. CapD has been demonstrated to be a *B. anthracis* virulence factor, contributing to virulence by anchoring the capsule to the peptidoglycan (Candela & Fouet, 2005) and by releasing low-molecular-mass capsule fragments that may interfere with innate immune function (Makino *et al.*, 2002). We previously showed that exogenous recombinant CapD efficiently degrades the capsule, resulting in increased susceptibility to phagocytic killing. Here we demonstrate that overexpression of *capD* in *B. anthracis* also results in increased susceptibility to phagocytic killing and decreased virulence *in vivo*, presumably due to cleavage of capsule from the bacillus surface by excess CapD, exposing the organism to innate immune defence mechanisms.

Despite the fact that *capD* resides on a single transcript with *capB*,*C* and *A*, its expression appears to be significantly lower than that of the other genes in the operon (Scorpio *et al.*, 2005). A previous report indicated that CapD was detected by immunoblots of crude extracts from *B. anthracis* RPG1 (Candela & Fouet, 2005). However, we were unable to detect CapD in *B. anthracis* Ames lysates with our polyclonal mouse anti-CapD antibody. The most recent *B. anthracis* genome annotation assigns the *capD* start codon as part of the *capA* stop codon, resulting in an overlap of the *capD* RBS sequence with the 3′ end of *capA*. Thus, translation of *capD* may be coupled with *capA* by a programmed shift of the ORF (Oppenheim & Yanofsky, 1980) in which the ribosome, upon completion of *capA* translation, is shifted back along the mRNA to reinitiate translation at the *capD* RBS sequence. The mechanism that suppresses *capD* expression may involve a combination of factors. For example, cistron terminal overlap (Shcherbakov & Garber, 2000) may affect *capD* translation efficiency due to ribosome competition between *capA* termination and *capD* initiation at the overlapping region (Gesteland & Atkins, 1996). Alternatively, *capD* expression may be regulated by a translational coupling mechanism in which the mRNA secondary structure inhibits ribosome priming (Rex *et al.*, 1994) at the *capD* translation initiation region. Finally, a weak RBS and TTG start codon may affect *capD* expression, although TTG start codons are common in Gram-positive bacteria and are reported to have minimal effect on translation efficiency (Ozbudak *et al.*, 2002). We created genetic fusions of *capD* to the 3′ end of the highly expressed genes *hsp60* and *EFTu* and integrated these into the chromosome of *B. anthracis*. Additionally, we created fusions of *capD* to the 3′ end of *capA* in which the TTG start codon was replaced with ATG and the putative RBS was replaced with the consensus sequence, AGGAGGT. The recombinant methods used to construct these strains resulted in a 6 bp separation (the *Xho*I cloning site) between the *capA* stop codon and the engineered *capD* RBS compared with the parent Ames strain, in which the *capD* RBS overlaps with the 3′ end of *capA* and is separated by 8 bp from the *capD* start codon. The engineered sequence changes resulted in higher expression of *capD* as measured by immunoblotting (Fig. 1a). In particular, adding a consensus RBS to *capD* when fused to *hps60* resulted in detectable CapD compared with no detection observed with the *hsp60capD* fusion having the wild-type RBS, suggesting that the RBS significantly affects *capD* translation, similar to the effect of RBS when comparing *capAcapD*RBSATG with *capAcapD*ATG (Fig. 1a).

Increased *capD* expression correlated with smaller amounts of surface-associated capsule, presumably due to degradation by CapD. Additionally, the culture supernatant capsule from *capD*-overexpressing strains in which CapD was detected by immunoblotting was found to be almost

entirely of low molecular mass. These phenotypic changes were associated with increased phagocytosis of bacilli by human neutrophils and murine macrophages, probably due to reduced surface-associated capsule. Overexpression of *capD* also reduced virulence in guinea pigs as measured by an increase in the mean survival time in a single-strain challenge and reduced fitness in a competitive index experiment comparing the wild-type Ames strain with *capAcapD*RBSATG. In the single-strain challenge with *capAcapD*RBSATG, bacilli isolated from the blood and spleen had a capsule that was not detectable by India ink but was visible by FITC-labelled monoclonal anti-capsule antibody stain, suggesting that only minimal surface capsule was required for dissemination from the site of infection for this strain (data not shown). The dissemination and residual virulence of the *capAcapD*RBSATG strain also supports the hypothesis that CapD-generated low-molecular-mass capsule contributes to the virulence of *B. anthracis* (Makino *et al.*, 2002). The higher amounts of low-molecular-mass capsule released from *capD*-overexpressing strains did not interfere with phagocytosis by neutrophils, as efficient killing of *capD*-overexpressing strains could be observed even at a 1 : 1 ratio of neutrophils to bacilli in which 10 % of the assay medium was replaced by supernatant capsule from the *capAcapD*RBSATG strain (data not shown). This suggests that the capsule associated with the bacillus surface is the primary mechanism for resistance to phagocytosis. It is possible that low-molecular-mass capsule may be important for resistance to other innate immune defence mechanisms such as cationic antimicrobial peptides and complement.

These data demonstrate that overexpression of *capD* resulted in significant capsule degradation during growth in BHI/bicarbonate broth and probably *in vivo*, resulting in smaller amounts of surface-associated capsule, an increase in released, low-molecular-mass capsule and reduced virulence. Taken together, the results suggest that fully virulent *B. anthracis* has evolved finely tuned expression of *capD* and that maximum virulence may require both wild-type levels of surface-associated capsule and released low-molecular-mass capsule.

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REFERENCES

Candela, T. & Fouet, A. (2005). *Bacillus anthracis* CapD, belonging to the gamma-glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. *Mol Microbiol* **57**, 717–726.

Candela, T., Mock, M. & Fouet, A. (2005). CapE, a 47-amino-acid peptide, is necessary for *Bacillus anthracis* polyglutamate capsule synthesis. *J Bacteriol* **187**, 7765–7772.

Chabot, D. J., Scorpio, A., Tobery, S. A., Little, S. F., Norris, S. L. & Friedlander, A. M. (2004). Anthrax capsule vaccine protects against experimental infection. *Vaccine* **23**, 43–47.

Day, W. A., Rasmussen, S. L., Carpenter, B. M., Peterson, S. N. & Friedlander, A. M. (2007). Microarray analysis of transposon insertion mutations in *Bacillus anthracis*: global identification of genes required for sporulation and germination. *J Bacteriol* **189**, 3296–3301.

Gesteland, R. F. & Atkins, J. F. (1996). Recoding: dynamic reprogramming of translation. *Annu Rev Biochem* **65**, 741–768.

Green, B. D., Battisti, L., Koehler, T. M., Thorne, C. B. & Ivins, B. E. (1985). Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infect Immun* **49**, 291–297.

Kimura, K., Tran, L. S., Uchida, I. & Itoh, Y. (2004). Characterization of *Bacillus subtilis* gamma-glutamyltransferase and its involvement in the degradation of capsule poly-gamma-glutamate. *Microbiology* **150**, 4115–4123.

Makino, S., Watarai, M., Cheun, H. I., Shirahata, T. & Uchida, I. (2002). Effect of the lower molecular capsule released from the cell surface of *Bacillus anthracis* on the pathogenesis of anthrax. *J Infect Dis* **186**, 227–233.

Menard, R., Sansonetti, P. J. & Parsot, C. (1993). Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J Bacteriol* **175**, 5899–5906.

Mendelson, I., Tobery, S., Scorpio, A., Bozue, J., Shafferman, A. & Friedlander, A. M. (2004). The NheA component of the non-hemolytic enterotoxin of *Bacillus cereus* is produced by *Bacillus anthracis* but is not required for virulence. *Microb Pathog* **37**, 149–154.

Mock, M. & Fouet, A. (2001). Anthrax. *Annu Rev Microbiol* **55**, 647–671.

Oppenheim, D. S. & Yanofsky, C. (1980). Translational coupling during expression of the tryptophan operon of *Escherichia coli*. *Genetics* **95**, 785–795.

Ozbudak, E. M., Thattai, M., Kurtser, I., Grossman, A. D. & Van, O. A. (2002). Regulation of noise in the expression of a single gene. *Nat Genet* **31**, 69–73.

Rex, G., Surin, B., Besse, G., Schneppe, B. & McCarthy, J. E. (1994). The mechanism of translational coupling in *Escherichia coli*. Higher order structure in the *atpHA* mRNA acts as a conformational switch regulating the access of de novo initiating ribosomes. *J Biol Chem* **269**, 18118–18127.

Richter, S., Anderson, V. J., Garufi, G., Lu, L., Budzik, J. M., Joachimiak, A., He, C., Schneewind, O. & Missiakas, D. (2009). Capsule anchoring in *Bacillus anthracis* occurs by a transpeptidation reaction that is inhibited by capsidin. *Mol Microbiol* **71**, 404–420.

Scorpio, A., Wei, J., Ghassemian, M., Su, J., Chen, W., Blank, T. E., Richardson, T. H., Bozue, J. A., Hoover, T. A. & other authors (2005). The proteome of *Bacillus anthracis* isolated from infected animals. In *Bacillus anthracis, B. cereus, and B. thuringiensis International Conference*, p. 20. Santa Fe, New Mexico, USA.

Scorpio, A., Chabot, D. J., Day, W. A., O'Brien, D. K., Vietri, N. J., Itoh, Y., Mohamadzadeh, M. & Friedlander, A. M. (2007). Poly-gamma-

glutamate capsule-degrading enzyme treatment enhances phagocytosis and killing of encapsulated *Bacillus anthracis. Antimicrob Agents Chemother* **51**, 215–222.

Scorpio, A., Tobery, S. A., Ribot, W. J. & Friedlander, A. M. (2008). Treatment of experimental anthrax with recombinant capsule depolymerase. *Antimicrob Agents Chemother* **52**, 1014–1020.

Shcherbakov, D. V. & Garber, M. B. (2000). Overlapping genes in bacterial and bacteriophage genomes. *Mol Biol (Mosk)* **34**, 572–583.

Su, J., Yang, J., Zhao, D., Kawula, T. H., Banas, J. A. & Zhang, J. R. (2007). Genome-wide identification of *Francisella tularensis* virulence determinants. *Infect Immun* **75**, 3089–3101.

Uchida, I., Makino, S., Sasakawa, C., Yoshikawa, M., Sugimoto, C. & Terakado, N. (1993). Identification of a novel gene, *dep*, associated with depolymerization of the capsular polymer in *Bacillus anthracis. Mol Microbiol* **9**, 487–496.

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